PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A61K 31/725

A1

(11) International Publication Number: WO 90/03791

(43) International Publication Date: 19 April 1990 (19.04.90)

(21) International Application Number: PCT/US89/04249

(22) International Filing Date: 27 September 1989 (27.09.89)

(30) Priority data:
253,686 5 October 1988 (05.10.88) US

(71)(72) Applicant and Inventor: DIFERRANTE, Nicola [US/US]; 1816 Norfolk, Houston, TX 77098 (US).

(74) Agent: MARSTELLER, Thomas, F.; P.O. Box 56265, Houston, TX 77256-6265 (US).

(81) Designated States: AT, AT (European patent), AU, BE (European patent), BR, CH, CH (European patent), DE, DE (European patent), DK, FR (European patent), GB, GB (European patent), IT (European patent), JP, LU, LU (European patent), NL, NL (European patent), SE, SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHODS FOR INTERFERING WITH HIV MULTIPLICATION AND COMPOSITION FOR THE TREATMENT OF AIDS

(57) Abstract

Heparin and low molecular weight derivatives of heparin lacking anticoagulant activity are effective in interfering with the life cycle of HIV. Cells exposed to heparin or its low molecular weight derivatives one hour before or after challenge with HIV showed no evidence of viral infection when examined four to six days post HIV challenge. Control cells similarly challenged with HIV showed 100 % HIV infection six days post HIV challenge. Cells challenged with HIV as much as 48 hours before exposure to heparin or its low molecular derivatives showed little evidence of HIV multiplication and evidenced healthy morphological features.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CM	Cameroon
DE	Germany, Federal Republic of
DK	Denmark
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
HU	Hungary
IT	Italy
JP	Japan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
LI	Liechtenstein
LK	Sri Lanka
LU	Luxembourg
MC	Monaco
MG	Madagascar
ML	Mali
MR	Mauritania
MW	Malawi
NL	Netherlands
NO	Norway
RO	Romania
SD	Sudan
SE	Sweden
SN	Senegal
SU	Soviet Union
TD	Chad
TG	Togo
US	United States of America

METHODS FOR INTERFERING WITH HIV MULTIPLICATION AND COMPOSITION FOR THE TREATMENT OF AIDS

SPECIFICATION

FIELD OF THE INVENTION

This invention relates to antiviral agents which interfere with viral intracellular multiplication.

BACKGROUND OF THE INVENTION

The progressive spreading of the AIDS infection with its tragic consequences on the world population has mobilized worldwide scientific efforts to contain the epidemic and search for effective means to alter the HIV virus life cycle.

The experience acquired in the past with other human viral diseases has immediately suggested that vaccines against the virus be considered and implemented. To this extent, either biosynthetic or extractive fragments of viral glycoprotein envelope, incorporated into appropriate biological carriers, have been injected into animals to assess whether they would be immunogenic (that is capable of causing production of antibodies) and whether the antibodies produced would interact with the virus and prevent its binding to the cells and receptors. Hopefully such an interaction would ameliorate the conditions of the affected subjects and would protect from infection those newly exposed to the virus.

As of now, these efforts have failed with respect to the HIV virus since the antibodies, when produced, have not been effective in arresting the viral spread nor the progression of the disease. This ineffectiveness may be caused, at least in part, by the high rate of mutation of the virus, with corresponding changes in the structure of all its gene products, including the envelope glycoproteins. Thus, a mutated virus may not be recognized by antibodies elicited by a slightly different antigen derived from a previous mutation.

The same arguments may apply to antibodies against the viral reverse transcriptase. This enzyme is responsible for the transcription of the viral genome - RNA - into the (corresponding) DNA which may initiate progeny formation or be incorporated in the genome of the host cell. Moreover, the difficulty of these antibodies to reach the intracellular compartments where the reverse transcriptase is active make their potential usefulness rather questionable.

Other approaches contemplate (1) the synthesis of those peptide fragments of the T-4 viral envelope glycoproteins which engage with the cell receptors prior to internalization of the whole virus; (2) the production of antibodies to the cell viral receptors; and (3) the biosynthesis of soluble fragments of T-4 cell viral receptors (CD4 antigen) capable of intercepting free, extracellular virus and binding it to prevent subsequent internalization. The first two products, by binding and occupying the viral receptors of the sensitive cells would make them unavailable for binding the virus. Considering the variety and the number of cells sensitive to viral attack present in a given organism, and the number of their viral receptors, one wonders whether those peptides or antibodies could be made available with a half-life and in amounts sufficient to keep the viral receptors constantly occupied for a finite amount of time.

Yet, approaches to interfere with the binding of the virus to the surface receptors of the sensitive cells are sound because, if successful, they would prevent the internalization of the virus which would remain exposed to the other humoral and cellular mechanisms of defense of the organism and would be prevented from incorporating its genome into that of the host cell.

However, such interference should be a permanent and complete one if it were to prevent healthy cells from becoming infected either from endogenous virus released by dying cells or exogenous virus acquired through the usual means of transmission. An inexpensive, easily available, low molecular weight, non-protein compound, capable of diffusing throughout the organism and capable of binding the virus or the viral receptors would be more suitable to this purpose than bulky peptides or proteins of finite half-life, costly preparation and potential immunogenic properties.

SUMMARY OF THE INVENTION

The present invention provides low molecular wight degradation products of naturally occurring linear polyanion heparin (glycosaminoglycan, acid mucopolysaccharide) lacking antithrombic and anticoagulant activities (i.e., less than 50 units antithrombin activity), yet retaining sufficient negative charges, as effective agents which interfere with the replication of the human immunodeficiency virus (HIV). The methods of invention provide for inhibiting T cell death and replication of human immunodeficiency virus (HIV) comprising exposing the human T cells to a pharmaceutically effective amount of a low molecular weight heparin degradation products lacking anticoagulant activity. The present invention further provides a method of treating HIV infection by administering to the infected host a therapeutically effective amount of a low molecular weight heparin degradation product

lacking anticoagulant activity. The administration can be accomplished through any of many routes including intravenously, intraperitoneally, orally, subcutaneously, intramuscularly, rectally, topically or by inhalation.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Heparin and Its Degradation Products.

Commercial heparin is prepared from beef lung or, preferentially from bovine or porcine intestinal mucosa by extraction procedures based on the high negative charge of molecule. After digestion of the crude material with proteases, the clear supernatant obtained by centrifugation is treated with quaternary ammonium salts. This precipitation may be achieved step-wise, at decreasing salt concentrations, the most highly charged molecules being obtained as a precipitate at the high salt concentration.

This higher charged heparin may be precipitated with quaternary ammonium salts above 0.35 M NaCl, while the other contaminating glycosaminglycans (chondroitin sulfates, heparan sulfate) remain in solution, to be precipitated upon further dilution of the solution.

The heparin-quaternary ammonium salt precipitate is repeatedly washed in the centrifuge with 95% ethanol/10% potassium acetate in order to remove the quaternary ammonium salt. The potassium salt of heparin is dissolved in water, cleared by centrifugation and further purified by column chromatography on anion or cation exchange resins.

Several methods are available for the isolation of heparin fractions of different molecular weight: a) anion exchange chromatography in which the heparin is retained on the column at low ionic strength and fractions of increasing molecular weight may be eluted by step-wise increase in the ionic strengths of the eluting solution (from 0.2 M to 3.0 M NaCl); or b) gel filtration, in which larger fractions emerge

first from the column, to be followed by gradually smaller ones. In this technique, the influence of sulfate content may be minimized by using as eluant a solution of 0.2 M NaCl.

The relevance of these techniques to the present invention depends on the linear correlation between molecular size and anticoagulant activity of heparin. We are particularly interested in heparin fractions lacking anticoagulant activity, yet still capable of interfering with the multiplication of the virus and still possessing the properties of stimulating cellular endogenous mechanisms of defense. Other methodologies that may be used for the same purpose include affinity chromatography of heparin on antithrombin-sepharose columns and factor Xa-sepharose columns in order to separate the high affinity fraction, having very high antithrombic or anticoagulant activity, from the low - affinity ones, essentially devoid of such activities. Thus, with these techniques, we were able to screen large and small molecular weight fractions of heparin without dangerous anticoagulant activity, for any possible inhibitory activity on the multiplication of the virus.

Additional chemical and enzymic methods may be used to degrade large molecular weight heparin fractions without further affecting their sulfate content. Dietrick, C.P., Biochem. J. 108: 647-654 (1968). Low molecular weight heparin (from porcine intestinal mucosa) is produced by nitrous acid degradation of native heparin, to a 4,000-6,000 MW. It is obtainable commercially through Sigma Company. This compound still possesses good anti-Xa activity, (> 150 IU/mg) but lower antithrombic activity (>40 IU/mg) than the undegraded original material. This low molecular weight heparin can be completely de-N-sulfated and N-acetylated as described below, in order to obtain a product without anticoagulant activity.

Heparin and heparan sulfate are glycosminoglycans which uniquely contain N-sulfate groups. Older methods of de-N-sulfation with controlled hydrolysis in dilute acids (0.04N HCl at 56° for 4 hours) caused hydrolysis of N-sulfated groups accompanied by some cleavage of glycosidic linkages (depolymerization) and of O-sulfate groups (de-O-sulfation). More recently, it has been reported that the pyridinium salt of N-sulfated glucosamine residues (present in heparin and heparan sulfate) is desulfated much more rapidly and selectively in dimethyl sulfoxide containing small amounts of water or methanol. The sodium salt or heparin (molecular weight 12,00 to 15,000) is dissolved in 25 ml of water and the solution is passed through a column of Dowex 50x8 at 4° C. The effluent and washing are combined, neutralized with pyridine and lyophilized to give the pyridinium salt of heparin.

This salt is dissolved in 100 ml of dimethyl sulfoxide containing 5% water and the solution is kept for 90 minutes at 50° C, then diluted with equal volume of water. The pH of the solution is adjusted to 9.0-9.5 with 0.1 M NaOH, dialyzed over night against tap water and then against distilled water for 20 hours. Filtration and lyophilization of the retentate gives the sodium salt of completely de-N-sulfated heparin. This large molecular weight compound (12,000 to 15,000 daltons) has no anticoagulant activity. This compound may be acetylated by the method of Danishefsky, et al, Arch. Biochem., Biophys. 90:114 (1960). 300 micrograms of the sodium salt of completely de-N-sulfated heparin in 30 ml of water are added to 5 ml of methanol and 0.9 ml of acetic anhydride. The mixture is kept for two hours at pH 6.5 by adding 10% $NaHCO_3$, under stirring at 3-4° C. The reaction mixture is dialyzed against running tap water over night and then against distilled water for 20 hours. Filtration and lyophilization of the retentate gives the sodium salt of

completely de-N-sulfated, N-acetylated heparin. If ^{14}C, ^{3}H or deuterium-labeled acetic anhydride is used, the final acetylated product will be conveniently labeled. Such a radioactively labeled derivative may be useful in following the fate of large molecular weight heparin added to a cell culture or administered to experimental animals.

Another facile and fruitful approach to the preparation of non anticoagulant derivatives of heparin is provided by E. Holmes et al., Hemostasis 16, Supplement 2: 1-7 (1986). Antithrombin (commercially available) bound to an insoluble matrix (Sepharose) may be used to separate large MW heparin into two fractions: one with high and one with low affinity for antithrombin (HA and LA respectively). The first has approximately 300 U anticoagulant activity/mg; the second is essentially inactive. This technique has allowed to identify the structure of the pentasaccharide of heparin responsible for binding to antithrombin. The internal glucosamine unit carries a O-sulfate group in position C-3, previously considered essential for interaction with antithrombin and absent in LA heparin. More recently, however, the consensus is that the 3-O-sulfate is an artifact produced during deamination of the 2-N-sulfate group, which migrates to position 3. In fact, the 3-O-sulfate group is never found in products of bacterial degradation of heparin. However, three additional sulfate groups, are required for high affinity to antithrombin. One of those, 2-N sulfate on the internal glucosamine, may be removed by solvolytic cleavage with methyl sulfoxide, leaving a highly sulfated, nonanticoagulant product.

Both these fractions (HA and LA) may be depolymerized with nitrous acid, to obtain fragments of various length which may be separated by gel chromatography. It is well documented that anticoagulant activity of heparin is highly dependent on molecular weight. Thus, oligosaccharides of less than 18 monosaccharides (less than 5,000 MW) length have

lost their anticoagulant activity but retained their anti-thrombic activity (anti-Xa activity).

Following the procedures of Pejlaer, G. et al., J. Biol. Chem. 263:5197 (1988), 42 micrograms of sodium nitrate ($NaNO_2$) are added to one microgram of heparin in 20 ml of ice-cold H_2O, acidified to pH 1.4 with dilute H_2SO_4. After three hours in the ice bath, the oligosaccharides are precipitated with 10 volumes ethanol and separated by gel chromatography on sephadex G-50. Fractions composed of 2 to 20 monosaccharides may thus be obtained. The nitrous acid treatment causes an oxidative deamination of N-sulfated glucosamine, with cleavage of the glucosaminidic linkage. Thus, N-sulfated glucosamine is transformed into 2,5-anhydromannose. This residue, located at the reducing end of the oligosaccharide products, may be conveniently reduced with sodium borohydride labeled with tritium (NaB^3H_4), to yield tritium-labeled mannose at the reducing end. This labeling technique allows one to follow the fate of these oligosaccharides[1], once added to a cell culture.

Finally, another method for degrading heparin to di and trisaccharides makes use of the enzyme heparinase II, obtained originally from Flavobacterium heparinum and now commercially available. 200 mg of heparin in 0.005 M phosphate buffer pH 7.0, containing 0.02% bovine serum albumin, is incubated at 25° C for up to 125 hours with heparinase II (10 units in 10 ml buffer). A series of disaccharides is obtained, which may be separated by high pressure liquid chromatography. Shorter periods of incubation, with the enzyme, may yield trisaccharides or oligosaccharides which may be separated by gel chromatography. All these products

---

[1] A note of caution: oligosaccharides prepared by nitrous acid oxidation have been used to produce monoclonal antibodies in mice. Thus, the possible antigenicity of 2,5 anhydromannose must be kept in mind. Pejlaer et al. J. Biol. Chem. 263:5197 (1988).

of enzymic cleavage have a 4-5 unsaturated uronic acid at the non-reducing end and have a MW of about 1,000 to about 3,000 daltons.

Heparin Sample B

Sample B used in these experiments is a degradation product of heparin (MW 4.000-5,000 daltons) prepared by mild oxidation of heparin with copper, H_2O2 and ascorbate.

100 g of sodium heparin were dissolved in 10 liters of a solution containing 0.5M NaCl; 0.35M sodium acetate; 0.35% ascorbic acid; 0.45% copper acetate and H_2O_2 to a final concentration of 1.8 volumes. The pH of the solution was adjusted to 7.80 before the addition of peroxide and again immediately after the addition of peroxide.

The solution was incubated at 50° C, with stirring for 20 hours. samples of 5-7 ml were collected at regular intervals. The buffered solution, prior to addition of peroxide and final pH adjustment, was pale blue-green in color. Upon addition of peroxide and alkali to pH 7.80, vigorous bubbling ensued, with a raise in the solution temperature to 65° C. The bubbling lasted for 30 minutes after which the temperature of the solution slowly returned to 50° C.

At the end of the incubation, the solution was clear and blue-green in color. The samples collected at various times during incubation and a final sample were processed in parallel throughout the following steps:

1. Passage through 7x1 cm columns of cation exchange resin Dowex 50x4, 200 mesh in the hydrogen form in order to retain the copper and allow recovery of free heparin in the effluent. Its elution was followed simply by monitoring the strong acidity of the effluent. Whenever the latter became neutral, the elution of heparin was complete.

2. Immediate neutralization of each heparin aliquot with sodium hydroxide, in order to obtain the sodium salt of the glycan.

3. The various aliquots were reduced in volume (if needed), with a rotary evaporator, under vacuum, and/or directly precipitated in the centrifuge with 2 volumes of absolute methanol. After washing twice with absolute methanol, each precipitate was washed and dried with ether to the powder state. The material obtained at the end of the incubation was weighed in order to calculate the yield of the process (80-85%).

4. Each sample collected during and at the end of the incubation was analyzed by electrophoresis on 6% acrylamide gels, to verify whether degradation had occurred. Samples of original and degraded heparin or Fragmin (a trademarked product of Kabi Vitrum) and of known products of heparin degradation were run as standards. The electrophoresis was run with 0.6M barbital buffer pH 8.60 for a period of one hour. Thereafter, the gels were stained with 0.1% Toluidine Blue in 1% acetic acid in water for 30 minutes. Destaining was carried out in 1% acetic acid, with mild agitation. Destained and dried gels were photographed or scanned to obtain a graphic representation of the degree of degradation.

5. When a final sample showed signs of great heterogeneity, with bands or peak indicating degradation products of different sizes, a water solution of the sample was passed through an appropriate size column of anion exchange resin Dowex 1x2, 100-200 mesh, in the chloride form in order to retain the heparin. This was eluted in discreet fraction of increasing molecular size using increasing concentration of NaCl: from 1.0M to 2.0,3.0 and 4.0M NaCl.

Because this techniques eluted the sodium salt of heparin, monitoring the effluent acidity was not feasible. Thus,

either one has to monitor the absorption of effluent at 310nm or spot a sample of effluent on filter paper impregnated with alcian blue in order to detect the methachromatic reaction of heparin.

The desired sample was then desalted, by gel filtration and precipitated with methanol and dried with ether as described in 3.

While in this series of experiments copper acetate was used as a source of metal catalyst, iron salts, copper or iron turning may be equally used for the production of OH free radicals (Fentón reaction).

Experimental

What follows is a description of the assays performed to demonstrate anti reverse transcriptase activity, if any, of

native heparin (Sample A) and Cu^{++}/H_2O_2/ascorbate degraded heparin (Sample B).

HIV RT ASSAY

10x Buffer:

1M Tris-HCL, pH 7.4-8.0	10 ul
1M DTT	10 ul
1M $MgCl_2$	8 ul
1M KCl	15 ul
10% Triton X-100	50 ul
6mM GSH	7 ul
	100 ul

Template:

10mM Tris + 0.1 mM EDTA	180 ul	
0.5mg/ml rAdT	120 ul	
Add H_2O	100 ul	
	400 ul*	(Dilute 1:2 Tris:EDTA)

RXN Mixture (1:5) (1:2)

10x Buffer	2.5 ul	5 ul
$[H^3]$-TTP:H_2O	5.0 ul	5 ul
Template	2.5 ul	5 ul
Sample*	15.0 ul	35 ul
	35.0 ul	50 ul

*Supernate of fluid plus cells centrifuged at 4°C.

1. Incubate RXN Mixture 2 hours at 41° C.
2. Spot 20 ul on DE81 strips (handle DE81 paper with gloves on only).
3. Wash with 5% Na_2HPO_4, 4-6x - 4 min/wash
   double distilled H_2O 2x - 1 min/wash
   95% EtOH 2x - 1 min/wash
4. Place in scintillation vials and Aquasol.

Example I

The virus, a source of reverse transcriptase, was used at three, decreasing concentrations, 1.0; 0.3; 0.1. Concentration 1 is arbitrary and it is defined, for the purpose of this assay, as that amount of virus which provides sufficient enzyme to cause incorporation of tritiated thymidine triphosphate (3_HTTP) into the template to the extent of 40,000 cpm. The two heparin preparations A and B have been used at several, decreasing concentrations: 1.0; 0.3; 0.1; 0.03 mg/ml.

The data indicate that both preparations cause inhibition of reverse transcriptase, preparation A better than B, with decreasing inhibition at decreasing concentrations. Virus dilution does not seem to enhance inhibitory activity.

Example II

This set of experiments investigated whether the two heparin preparations (A or B) interfere with the multiplication of HIV in T cells. Both heparin preparations were added to the cells either 1 hour before the virus was added or 1 hour after the virus was added. The rationale for this is that we did not know whether heparin might gain access to the cell interior by itself or whether it needs the virus to proceed - piggy-back - therein. (See, "Animal Viruses Promote the Entry of Polysaccharides with Antiviral Activity into Cells," E. Gonzalez E. L. Carrasco. Biochem. Biophys. Res. Commun. 146; 1303-1310, 1987).

In these experiments T cells derived from human adult lymphocytic leukemia (ALL), line C8166, were used at a concentration of 2 x 10^5cells in 0.5 ml of medium RPMI1640 (standard medium for such experiments). This amount of cells was place in each well of a multi-well plate. 0.1 ml of HIV virus suspension (HIV pp213, stock of UTMB Galveston) was

added to each well and incubation of the plate was carried out at 37° C for up to 21 days.

The concentration of the virus (1:10 dilution from the stock solution) was adopted so as not to kill the cells immediately but nevertheless to provide total infection of the cells within 4-5 days.

In a first set of experiments, cells were exposed to the heparin preparations A or B by adding the heparin sample to the cells 1 hour before or 1 hour after challenging with virus.

Wells were examined every 2 days and samples from wells were removed to measure cellular presence of a viral p-24 core protein antigen. Cells from these samples were spotted in the microscopic fluorescence slides (Cell Line Associates, Inc. 33 Cargo line, Newfield, NJ, fluorescence slides, 8 wells, 8mm), and fixed with cold acetone and treated with a fluorescent monoclonal antibody against P24. The fluorescent cells in each sample were counted and expressed as a percentage of all cells, using a fluorescence microscope.

TABLE 1: HEPARIN ADDED 1 HOUR BEFORE VIRUS

Concentrations/ml

HEPARIN A	No Heparin	1mg	0.3mg	0.1mg	0.03mg
DAY 4	30% pos	neg	neg	neg	1-2% pos
DAY 6	100% pos	neg	neg	neg	1-3% pos
HEPARIN B					
DAY 4	30% pos	neg	neg	neg	neg
DAY 6	100% pos	neg	neg	neg	neg

TABLE 2: HEPARIN ADDED 1 HOUR AFTER VIRUS ADDITION

HEPARIN A	No Heparin	1mg	0.3mg	0.1mg	0.03mg
DAY 4	30% pos	neg	neg	neg	1-3% pos
DAY 6	100% pos	neg	neg	neg	1-3% pos
HEPARIN B					
DAY 4	30% pos	neg	neg	neg	neg
DAY 6	100% pos	neg	neg	neg	neg

CONTROLS: Cells without virus, all negative.

Cells without virus but with heparin A or B at stated concentrations, to investigate for toxicity, as per visual morphological inspection, revealed that Sample A showed +/- toxicity at 1mg concentration only, while Sample B did not show toxicity at any concentration.

EXAMPLE II

An additional experiment was performed with Heparin B, to establish any effect on late infection. The test was designed essentially along the lines described in EXAMPLE II. The same cell line, same HIV virus strain, same fluorescence technique, were used to detect P-24 antigen on cell surface. The differences included: 10 the viral inoculum was carried out with full strength virus concentration, in order to obtain in the controls the full effect of the infection in a short period of time; and 2) the Heparin B was added 48 hours later. The virus killing effect was measured by the tripan blue exclusion method. The rest of the experiment was the same. Wells were examined every day for morphological evidence of cytopathic effects (CPE) (*) and cell samples were obtained every day for fluorescence microscopy to identify presence of P-24 on cell surface (#). Heparin B was used at 1.0mg; 0.3mg; 0.1mg and 0.03mg/ml, as previously noted. Both

the cytopathic effects and the amount of fluorescence were scored from 1+ to 4+.

TABLE 3: CPE (*=0) AND FLUORESCENCE (#=0)

Days of experiment	0	1	2	3	4	5	6	7	8	9	10
Control cells, not infected	*	*	*	*	*	*	*	*	*	*	*
	#	#	#	#	#	#	#	#	#	#	#
Control cells, + Heparin B for each of 1.0; 0.3; 01.; and 0.03mg/ml	*	*	*	*	*	*	*	*	*	*	*
	#	#	#	#	#	#	#	#	#	#	#
Control cells infected	*	*	*	1+	3+	3+					
	#	#	3+	3+	3+	4+					
Cells infected + Heparin B for each of 1.0;; 0.3; 0.1; 0.03mg/ml	*	*	*	*	*	*	*	*	*	*	*
	#	#	3+	3+	3+	3+	3+				

It is clear that Heparin B protects the cells from being killed by the virus even though they had been infected for 48 hours. Control cells, infected but not treated show morphological changes and fluorescence by day 2 and 3, with cell death by day 5.

Control cells plus Heparin B do not show changes of toxicity. However, cells infected at time 0 and treated 48 hours later do not show cytopathic effects, even though they display the presence of viral core protein (P-24).

These data indicate that Heparin B, administered 48 hours after viral infection does not prevent the synthesis of the core P-24 antigen of the virus. The latter apparently

must have had time to shed the glycoprotein coat, to free its RNA from the core protein, to have the RNA transcripted into DNA and this incorporated into host DNA.

The presence on the cell of P-24 antigen clearly indicates that the virus has undergone a good part of its intracellular life cycle. Yet, the treated cells do not show signs of suffering, and survive well. Thus, Heparin B apparently acts quite late in the life cycle of the virus, protecting cells from virus killing.

While the presently preferred embodiments of the invention have been set forth for the purposes of disclosure, changes and modifications therein can be made which are within the spirit of the invention as defined by the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method of inhibiting the multiplication of HIV in human T cells comprising exposing the human T cells to a pharmaceutically effective amount of low molecular weight heparin degradation product lacking anticoagulant activity, said heparin degradation product obtained by oxidation of heparin using free radical OH.

2. The method of Claim 1 wherein the heparin degradation product has a molecular weight ranging from about 4,000 to about 6,000 daltons.

3. The method of claim 1 wherein the heparin degradation product is prepared by mild oxidation of heparin using H_2O_2 and a metal catalyst.

4. The method of claim 1 wherein the pharmaceutically effective amount ranges from about 1mg/ml to about 0.03 mg/ml.

5. A method of prolonging the survival time of human T cells challenged with the human immunodeficiency virus (HIV) comprising exposing the human T cells to a pharmaceutically effective amount of low molecular weight heparin degradation product lacking anticoagulant activity, said heparin degradation product obtained by oxidation of heparin using free radical OH.

6. The method of claim 5 wherein the heparin degradation product has a molecular weight ranging from about 4,000 to about 6,000 daltons.

7. The method of claim 5 wherein the heparin degradation product is prepared by mild oxidation of heparin.

8. The method of claim 5 wherein the pharmaceutically effective amount ranges from about 1mg/ml to about 0.03mg/ml.

9. A method of treating a host infected with human immunodeficiency virus (HIV) comprising administering to the host a pharmaceutically effective amount of a low molecular weight heparin degradation product lacking anticoagulant activity, said heparin degradation product obtained by oxidation of heparin using free radical OH.

10. The method of claim 9 wherein the heparin degradation product has a molecular weight ranging from about 4,000 to about 6,000 daltons.

11. The method of claim 9 wherein the heparin degradation product is prepared by mild oxidation of heparin.

12. The method of claim 9 wherein the pharmaceutically effective amount ranges from about 1mg/ml to about 0.03mg/ml.

13. The method of claim 9 wherein the heparin degradation product is administered orally, intravenously, or intraperitoneally, subcutaneously, intramuscularly, topically, rectally or by inhalation.

14. A pharmaceutical composition for treating human immunodeficiency virus (HIV) infection comprising:

a pharmaceutically effective amount of a low molecular weight heparin degradation product lacking anticoagulant activity together with a pharmaceutically acceptable diluent, said

heparin degradation product obtained by oxidation of heparin using free radical OH.

15. The pharmaceutical composition of claim 14 wherein the heparin degradation product has a molecular weight ranging from about 4,000 to about 6,000 daltons.

16. The pharmaceutical composition of claim 14 wherein the heparin degradation product is prepared by mild oxidation of heparin.

17. The pharmaceutical composition of claim 14 wherein the pharmaceutically effective amount ranges from about 1mg/ml to about 0.03mg/ml.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/04249

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K31/725

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C08B ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
Y	WO,A,8606729 (OPOCRIN) 20 November 1986 see abstract	14-17
Y	Proceedings of the National Academy of Science, USA vol. 85, no. 16, August 1988, (Washington, US) pages 6132 - 6136; M. Baba et al.: "Mechanism of inhibitory effect of dextran sulfate and heparin on replication of human immunodeficiency virus in vitro" see abstract and table 3	14-17

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11 JANUARY 1990	12. 02. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	T.K. WILLIS

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 1-13 because they relate to subject matter not required to be searched by this Authority, namely:

PCT Rule 39.1(iv):
Methods for treatment of the human or animal body
by surgery or therapy, as well as diagnostic methods.

2. [ ] Claim numbers....., because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- [ ] The additional search fees were accompanied by applicant's protest.
- [ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/US 89/04249

SA 31860

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 03/02/90

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-8606729	20-11-86	EP-A- 0221977	20-05-87
		JP-T- 63500184	21-01-88

EPO FORM P0479

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82